Specific benzodiazepine receptors in rat brain characterized by high-affinity [3H]diazepam binding

(affinity binding/diazepam/anxiolytic activity/brain membranes/regional distribution)

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ABSTRACT [3H]Diazepam appears to bind specifically to a single, saturable, binding site located on rat brain membranes, with an affinity constant near 3 nM at pH 7.4. Specific binding constitutes more than 90% of total binding at 0° and less than 10% of total binding at 37°. Arrhenius plots suggest a sharp conformational change in the diazepam receptor near 18°. Mitochondrial fractions from rat kidney, liver, and lung exhibit some [3H]diazepam binding that can be displaced by nonradioactive diazepam and several other benzodiazepines. However, Ro-4864, which is almost inactive in displacing [3H]diazepam from brain membranes, is extremely potent in displacing it from kidney mitochondria. Conversely, clonazepam, the most potent inhibitor of brain binding, is an extremely weak inhibitor of kidney binding. Furthermore, diazepam binding to kidney mitochondria has an affinity constant of 40 nM, about 15 times higher than that in brain. No specific diazepam binding was detected in intestine or skeletal muscle. Thus, specific [3H]diazepam binding to membranes appears to be restricted to brain, where it is unevenly distributed: the density of diazepam receptors is about five times higher in cortex (the highest density) than in pons-medulla (lowest density). Trypsin and chymotrypsin completely abolished specific [3H]diazepam binding in brain and kidney.

The exact neurochemical mechanism of action of the benzodiazepine minor tranquillizers remains unknown although several models have recently been proposed (1–3). Recently, we demonstrated that [3H]diazepam specifically binds to rat brain membranes with high affinity (4) and that the activity of 20 benzodiazepines in preventing this binding correlated well with their activity in several pharmacological tests predictive of clinical effectiveness in man (C. Braestrup and R. F. Squires, unpublished data). The present report describes some properties of the specific diazepam receptor in brain, as well as distinct diazepam receptors in mitochondria from rat kidney, skeletal muscle, and lung.

MATERIALS AND METHODS

[*N-methyl-3H*]Diazepam (14.4 Ci/mmol) was kindly provided by Willy Haefely, F. Hoffman-La Roche and Co. AG, Basel, and was stored in absolute ethanol at −20° to prevent radiolysis. This stock solution was diluted (1:10,000) in 50 mM Tris · HCl, pH 7.4, to constitute a working solution which was stable for at least 7 days at 4°. Lorazepam and oxazepam were kindly donated by Wyeth Laboratories and Librium (chlordiazepoxide), clonazepam, and Ro-4864 by Hoffmann-La Roche. Other drugs and materials were obtained from commercial sources.

For the [3H]diazepam binding assay, male Wistar rats (Ferrosan Laboratories, Copenhagen), 200–250 g, were de-

capitated and their brains were removed immediately. The whole forebrain was excised and homogenized in 20 volumes of iced 0.32 M sucrose in a Potter-Elvehjem homogenizer fitted to 0.25 mm clearance. The homogenate was centrifuged for 5 min at 2000 × *g* at 5° and the supernatant was recentrifuged for 10 min at 30,000 × *g*. The pellet from the second centrifugation (P_2 pellet) was resuspended in 50 times the original tissue weight of 50 mM Tris · HCl, pH 7.4, and used directly in the binding assays. Five hundred microliters of this P_2 suspension (corresponding to 10 mg of original tissue) was preincubated for 5 min; then, 25 μl of [3H]diazepam working solution (to give a final concentration of *ca* 2 nM or approximately 14,000 cpm) was added and the incubation was continued at 37° for an additional 15 min. The samples were then cooled in an ice bath for 30 min. Ten milliliters of iced 50 mM Tris · HCl, pH 7.4, was added to each sample immediately before it was filtered through Whatman GF/C glass fiber filters. The filters were washed immediately with an additional 10 ml of iced buffer, shaken thoroughly with 12 ml of scintillation cocktail [1 volume H_2O/2 volumes Triton X-100/4 volumes toluene/0.04% (wt/wt) 2,5-diphenyloxazole, and 0.01% 1,4-bis[2(5-oxazolyl)]benzene in a counting vial, and assayed for 3H.

Experiments were always run in series with at least four control samples, duplicate or triplicate blanks containing 3 μM diazepam, and test samples in duplicate or triplicate. Specific binding is defined as the difference between total binding and binding in the presence of excess diazepam or other active benzodiazepines (nonspecific binding). Nonspecific [3H]diazepam binding was about 7–9% of total binding. Blank values obtained with an excess of other benzodiazepines such as lorazepam, oxazepam, and chlordiazepoxide were consistently a little higher (12–14% of total binding) than diazepam blanks.

In some experiments we used a cell membrane fraction for the binding assay instead of the P_2 suspension described above.

The P_2 pellet was osmotically shocked by suspending it in 20 volumes of water (pH 8) for 15 min at 0°. The suspension was then centrifuged for 10 min at 8000 × *g*, and membranes were isolated from the supernatant by centrifugation for 10 min at 30,000 × *g*. The pellet, called the "membrane fraction," was reconstituted in 20 times the original tissue weight of 50 mM Tris · HCl, pH 7.4, and used for binding assays.

RESULTS

Binding as a Function of Tissue Concentration, Incubation Time, and Temperature. Specific binding increased linearly with increasing tissue concentration over the range of 0–20 mg of original tissue per assay. The linear range appeared to be somewhat increased when we used GF/B filters (0.7 mm thick) instead of GF/C (0.35 mm thick) (Fig. 1) or two GF/C filters
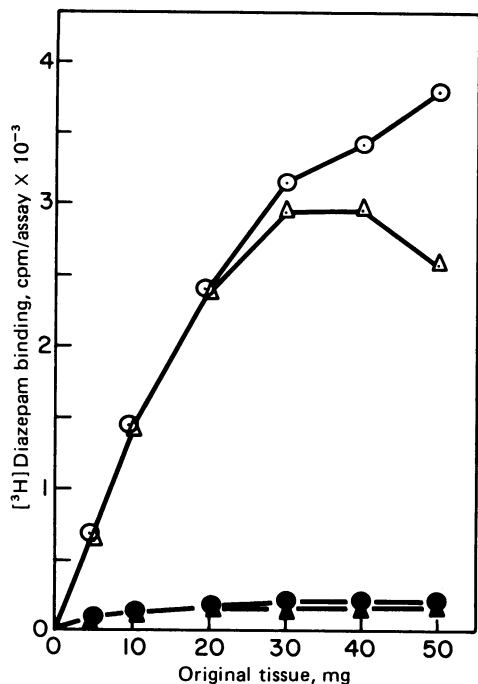
FIG. 1.   Effect of increasing amount of tissue on [³H]diazepam binding to rat brain P_2 suspensions, in 0.5 ml of Tris·HCl buffer. Either GF/C (△) or GF/B (○) filters were used. Open symbols, specific binding; solid symbols, nonspecific binding. The experiment was repeated three times with similar results.

(not shown). Filtration through GF/C filters was faster than through GF/B filters. Assays were routinely conducted with GF/C filters and 10 mg of tissue per assay, which is well within the linear range.

Diazepam binding equilibrium was reached within 10–15 min at 0° (Fig. 2). Preincubation at 37° is therefore not required to obtain rapid binding equilibrium. However, maximal binding was found to be increased by 10–30% after preincubation at 37°, compared to incubation at 0° alone. In contrast, preincubation of rat kidney mitochondria for 15 min at 37° resulted in a decrease in specific [³H]diazepam binding.

Incubation at 37° for 15 min without subsequent cooling

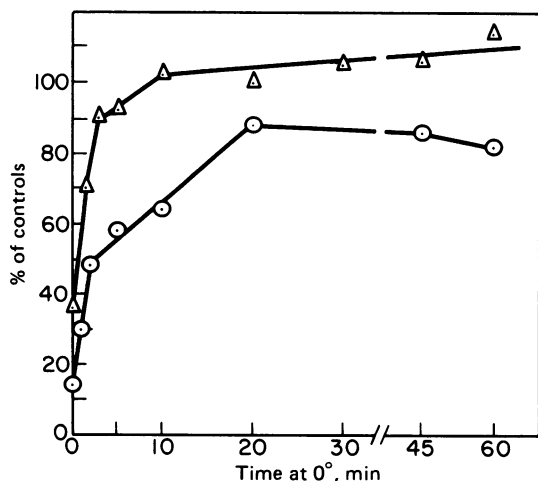FIG. 2.   Time course of specific [³H]diazepam binding to rat brain P_2 suspensions at 0°. △, Samples initially incubated at 37° as described in the general procedure; ○, samples incubated only at 0°. Mean (±SEM) specific binding in controls was 1580 ± 62 cpm/assay (n = 7) and nonspecific binding was 175 ± 9 cpm/assay.
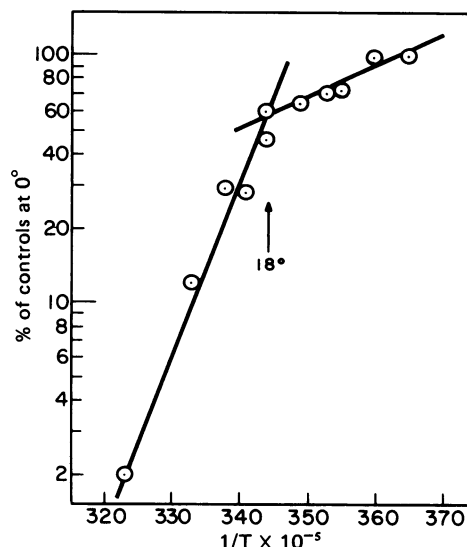

FIG. 3.   Temperature dependence of [³H]diazepam binding to rat brain membranes. Samples were incubated in triplicate at temperatures between 0° and 37° for 30 min and then were filtered directly after incubation and washed with buffer at the same temperature. Mean (±SEM) control specific binding was 723 ± 52 cpm per assay (n = 6); nonspecific binding was about 90 cpm per assay. Data are plotted according to Arrhenius.

yielded only 2% of the specific binding obtained at 0°. Equilibrium binding at different temperatures (0°–37°) are presented as Arrhenius plots in Fig. 3. The break on the curve indicates that the binding site for [³H]diazepam undergoes a conformational change at about 18°.

Membrane Fractionation. Cell membranes from the osmotically shocked synaptosome pellet (P_2) were fractionated by differential centrifugation. Fig. 4 shows that monoamine oxidase activity, with kynuramine as substrate (5), sedimented at 4000 and 8000 × g, as expected of an enzyme localized in mitochondria. Maximum [³H]diazepam binding occurred in
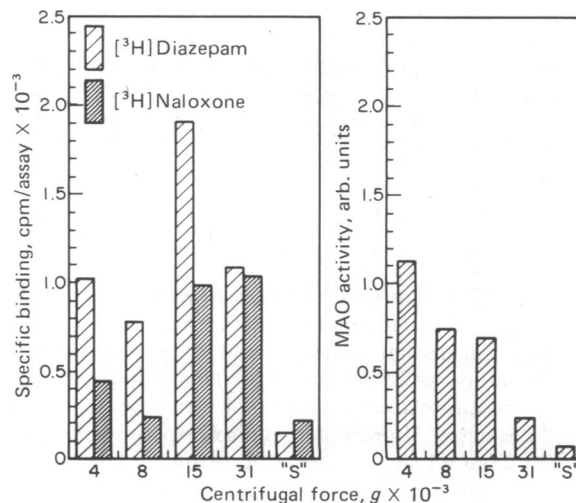

FIG. 4.   Differential centrifugation. The P_2 pellet from rat brain was resuspended in water, osmotically shocked for 15 min at 0°, pH 8, and then subjected to consecutive 10-min centrifugations of increasing force. The pellets were resuspended in 20 times the original tissue volume and assayed for specific [³H]diazepam binding, specific [³H]naloxone binding, and monoamine oxidase (MAO) activity. "S" denotes the final supernatant. Nonspecific [³H]diazepam binding was 80–90 cpm per assay. The experiment was performed four times with similar results.

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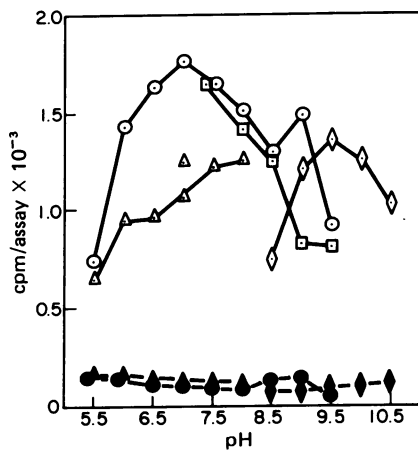
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FIG. 5. Specific [³H]diazepam binding to rat brain P₂ suspensions at different pH in different buffers. At high pH values, the base was 50 mM and adjusted with acid; at low pH values, the acid was 50 mM and adjusted with base. O, Tris-phosphate; △, Tris-citrate; □, Tris-HCl; ◇, glycine/NaOH. Open symbols, specific binding; solid symbols, nonspecific binding.

the 15,000 × g fraction where cell membranes probably sediment. In addition, we monitored specific [³H]naloxone binding (4.5 nM) (C. Braestrup and R. F. Squires, unpublished data) for comparison. Both [³H]naloxone binding and [³H]diazepam binding occurred in the membrane fractions sedimenting between 8000 and 30,000 × g. It is of interest that in four separate experiments the [³H]diazepam receptor sedimented at a lower centrifugal force than did the [³H]naloxone binding site. This shows that [³H]diazepam and [³H]naloxone binding do not occur in identical membrane fractions.

pH Optimum. Specific [³H]diazepam binding displayed a rather broad pH optimum, with no great variations in the amount of binding between pH 6.5 and 9 (Fig. 5). Tris-phosphate buffer yielded higher binding than Tris-citrate while glycine/NaOH displaced the pH optimum to about 9.5.

Stability. Specific diazepam binding was stable. Brain tissue or isolated membranes were kept frozen at −18° for 7 days without loss of activity and the P₂ suspensions in Tris-HCl, pH 7.4, were stable overnight at 3° without loss of activity. Heat inactivation of the receptors at 50° and 60° revealed half-lives of about 3 hr and 10 min, respectively (Fig. 6). We found that
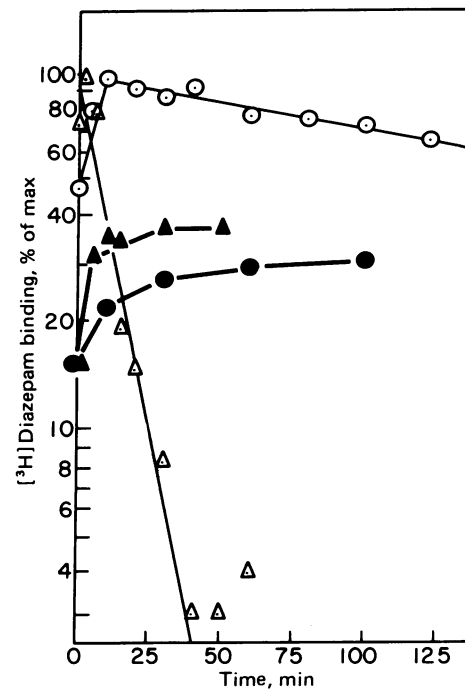

FIG. 6. Inactivation of [³H]diazepam binding sites on rat brain membranes by preincubation at 60° (△) and 50° (O). Open symbols, specific binding; solid symbols, nonspecific binding. Mean (±SEM) maximal specific binding was 810 ± 50 cpm per assay (n = 4). The experiment was repeated twice with similar results.

incubation at 50° or 60° invariably resulted in an initial increase in specific binding. This phenomenon has been observed by others for the opiate receptor and has been ascribed to the presence of an endogenous ligand (6).

When the P₂ suspension was incubated for 20 min at 37° at pH 11 (10 mM borate buffer) and then brought back to pH 7.4, no loss of activity occurred. Incubation for 20 min at 37° at pH 2 (10 mM HCl) resulted in almost complete loss of specific binding but nonspecific binding was unchanged.

Saturation of [³H]Diazepam-Binding. Specific binding was saturable, with half-maximal binding at about 3 nM (Fig. 7). Nonspecific binding increased linearly with [³H]diazepam concentration. Scatchard analysis of the data indicated a single
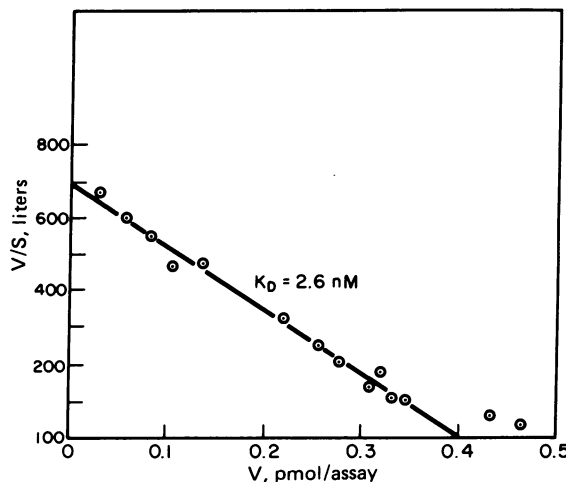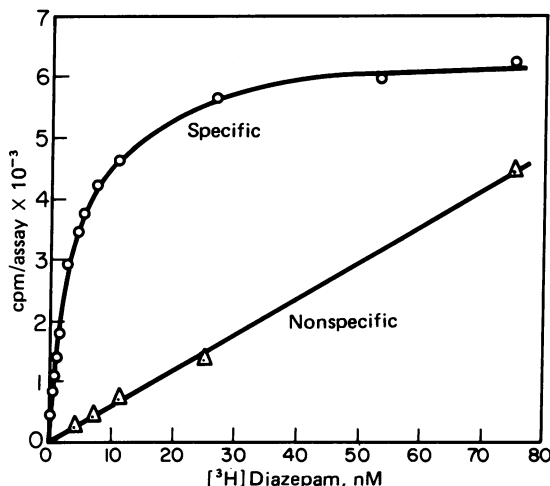

FIG. 7. (Left) Binding to rat brain P₂ suspensions of [³H]diazepam in concentrations from 0.24 to 70 nM. (Right) Scatchard analysis of the same data, indicating one binding site. All samples were incubated in triplicate. The whole experiment was repeated several times with similar results.

Table 1.   Regional distribution of [³H]diazepam binding to P₂ suspensions from rat brain

Region	Weight, mg	Specific binding, cpm/g tissue × 10⁻³	Blank, % of total binding
Frontal cortex	48	161 ± 45	8
Parietal cortex	83	150 ± 80	9
Occipital cortex	143	148 ± 27	9
"Rest"	423	130 ± 18	11
Limbic forebrain	88	127 ± 18	10
Hippocampus	100	110 ± 15	12
Hypothalamus	115	78 ± 22	18
Corpus striatum	73	71 ± 15	19
Cerebellum	230	51 ± 16	19
Pons medulla	183	32 ± 7	35

Provided that no great variation in affinity constants exists among the different regions, a notion supported by the single binding component suggested by Fig. 7 *right*, the binding shown in this table, obtained with 1.6 nM [³H]diazepam, reflects the relative receptor densities. The results are the means of two separate experiments, each conducted in quadruplicate.

binding component with an apparent affinity constant of 2.6 nM. The total number of binding sites was 18 pmol/g of original rat whole brain tissue. Use of increasing concentrations of [³H]diazepam up to 900 nM did not indicate the presence of other specific binding sites.

Regional Distribution of [³H]Diazepam Binding. Marked regional variations in specific [³H]diazepam binding existed (Table 1) and failed to parallel the regional distribution of γ-aminobutyric acid (7), glycine (7), substance P (8, 9), endorphins or opiate receptors (10–12), neurotensin (13) or dopamine, norepinephrine, serotonin, and acetylcholine.

Trypsin Digestion. Preincubation of P₂ synaptosome suspensions for 30 min at 37° and pH 7.4 with trypsin (10 μg/ml, Sigma) or chymotrypsin (100 μg/ml, Sigma) resulted in complete loss of specific [³H]diazepam binding capacity. Trypsin inhibitor (100 μg/ml, Sigma) had no effect on binding.

Brain Specificity. [³H]Diazepam binding was investigated in particulate fractions from several tissues. Skeletal muscle and small and large intestine exhibited no specific binding; some specific binding was observed in kidney, liver, and lung (Table 2). Scatchard analysis of the binding to kidney (not shown) indicated an affinity constant of 40 nM, about 15 times higher than that for brain. The displacement characteristics of the very

Table 2.   [³H]Diazepam binding to different organs

Tissue	Specific binding, as % of brain tissue	Binding, cpm/assay Total	Binding, cpm/assay Nonspecific
Kidney	39	752 ± 71	141 ± 3
Liver	15	540 ± 118	302 ± 44
Lung	10	297 ± 137	146 ± 78
Small intestine	0	244 ± 43	245 ± 3
Large intestine	1	122 ± 17	101 ± 6
Muscle	0	124 ± 37	138 ± 8
Brain	100	1764 ± 147	190 ± 10

All tissues were homogenized with an Ultra Turrax homogenizer. The P₂ pellet was resuspended in 25 times the original volume of Tris·HCl (50 mM, pH 7.4) for assay of [³H]diazepam binding (1.6 nM). The results are the mean ± SEM of one experiment in quadruplicate; the experiment was repeated with similar results.

Table 3.   Displacement of specific [³H]diazepam binding in different tissues by two benzodiazepines

Tissue	IC₅₀, nM Clonazepam	IC₅₀, nM Ro-4864
Kidney	2900 ± 600 (4)	4.7 ± 0.3 (4)
Liver	5080 (1)	4.1 (1)
Lung	7900 (1)	4.8 (1)
Brain	5.0 (2)	163,000 (2)

IC₅₀ values are the concentrations producing 50% inhibition of specific [³H]diazepam binding (1.6 nM) in assays performed as described in Table 2 with three or four concentrations of the inhibitor in duplicate. Values in parentheses are numbers of experiments.

potent anxiolytic benzodiazepine, clonazepam, on the one hand and the almost inactive benzodiazepine, Ro-4864, on the other clearly differentiated [³H]diazepam binding to brain from binding to kidney, liver, and lung. Displacement of brain binding paralleled clinical efficacy, clonazepam being extremely potent and Ro-4864 being almost devoid of activity, whereas binding to kidney, liver, and lung exhibited exactly the opposite specificity (Table 3).

Furthermore, [³H]diazepam binding in kidney homogenates was associated with a fraction sedimenting with mitochondria, in contrast to the association of [³H]diazepam binding with a membrane fraction in brain.

DISCUSSION

The results presented here demonstrate a specific diazepam receptor in rat brain membranes. The receptor has not been detected in any other tissues of the rat examined so far. Binding is saturable, has high affinity, and has an uneven regional distribution. The binding site is probably a rather stable protein localized in cell membranes.

Specific binding of [³H]diazepam does occur to receptors in kidney, liver, and lung. However, this binding appears to be fundamentally different from the specific binding to brain membranes: first, the displacement characteristics of binding to kidney, liver, and lung are entirely different; second, binding in kidney is associated with a mitochondrial fraction while that in brain is associated with a membrane fraction; and third, the affinity of [³H]diazepam for its receptor in kidney is about 15 times less than its affinity for the brain binding site. Serum albumin has also been shown to bind various benzodiazepines with relatively low affinity (14) but, like the binding of [³H]-diazepam to kidney, liver, and lung described here, this binding to serum albumin is clearly different pharmacologically from the binding to brain membranes.

Presumed neurotransmitters such as dopamine, norepinephrine, serotonin, acetylcholine, γ-aminobutyric acid, glycine, substance P, and endorphins have no affinity for the benzodiazepine receptors (ref. 4; C. Braestrup and R. F. Squires, unpublished data). It seems, therefore, that the benzodiazepines do not act directly on any of the known neurotransmitter receptors, a notion further supported by the regional distribution which does not parallel any presumed neurotransmitter.

It is well known that a compound may exhibit binding in both a saturable and a stereospecific way without any physiological or pharmacological relevance (15). Pharmacological studies with the [³H]diazepam binding site in brain tissue have shown that it is selective for benzodiazepines and that the ability of different benzodiazepines to displace [³H]diazepam is remarkably well correlated with those pharmacological effects of benzodiazepines that are thought to be predictive of anxiolytic activity in man (4).

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We therefore conclude that the binding site for [^{3}H]diazepam is physiologically relevant and that an unknown endogenous transmitter acting on these receptors may exist.

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